

HIGH-RESOLUTION PROTON NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY OF CYTOCHROME *c**

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Abstract.—In cytochrome *c* the axial positions of the heme iron are occupied by two amino acid residues, one of which is known from X-ray studies to be histidyl. Nuclear magnetic resonance spectroscopy provides strong evidence that the sixth ligand is a methionyl residue in both the ferric and ferrous oxidation states. It is further shown that in cytoferricytochrome *c* cyanide ion replaces methionyl in the first coordination sphere of the heme iron. Additional data are obtained on the protein conformation and on the electronic structure of the heme group in ferricytochrome *c*. As in other heme proteins, the interactions with the polypeptide chain greatly affect the unpaired electron distribution in the heme group of cytochrome *c*. In particular, from a comparison of ferricytochrome *c* and cytoferricytochrome *c*, the importance of the coordination of the sixth ligand is apparent.

Introduction.—Cytochrome *c*¹ is a protein of the respiratory chain which contains one heme group (Fig. 1) per molecule. The axial positions of the heme iron are occupied by two amino acid residues, one of which was found from X-ray studies to be the histidyl residue in position 18.² The present nuclear magnetic resonance (NMR) experiments yield information on the sixth ligand (Fig. 1). In the biological role of cytochrome *c*, interconversion between the ferric and ferrous oxidation states of the heme iron is an important factor. This paper presents a preliminary discussion of conformational changes arising from interconversion between the ferric and ferrous oxidation states, and of the electronic structure of the heme group in ferricytochrome *c*.

Nuclear magnetic resonance studies of cytochrome *c* have been described previously. Kowalsky³ reported that hyperfine interactions with the heme iron give rise to large upfield and downfield shifts of three proton resonances of ferricytochrome *c*. More recently, McDonald and Phillips studied the denaturation of cytochrome *c*⁴ and presented an interpretation of the NMR spectrum of ferrocytochrome *c*.⁵

Experimental.—Ferricytochrome *c* of Guanaco was obtained from Dr. E. Margoliash. For the NMR experiments, *ca.* 0.01 *M* solutions in 0.1 *M* deuterated phosphate buffer, pD 7.0, were prepared. Cytoferricytochrome *c* was prepared by addition of KCN. Ferrocytochrome *c* was obtained by reduction of ferricytochrome *c* with ascorbic acid or disodium-dithionite.

High-resolution proton NMR spectra were recorded on a Varian HR-220 spectrometer equipped with a standard Varian variable-temperature control unit. The temperature in the sample zone was determined from the chemical shifts of the resonances of ethylene glycol. Chemical shifts are expressed in parts per million (ppm) from internal DSS (sodium 2,2-dimethyl-2-silapentane-5 sulfonate), where shifts to low field are assigned negative values.

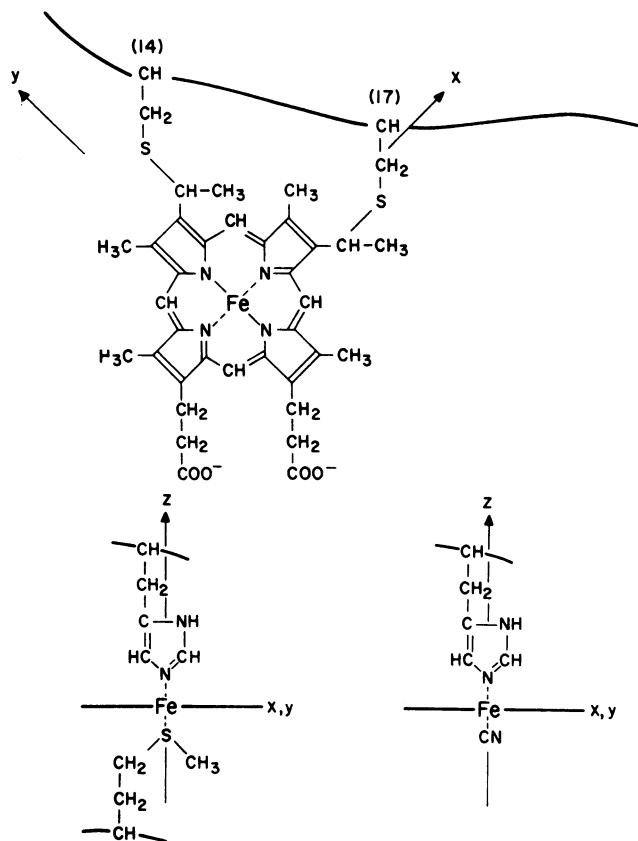


FIG. 1.—Heme group of cytochrome *c* and the axial ligands of the iron in cytochrome *c* and its complex with cyanide ion. In place of the methionyl residue, arginyl and lysyl residues have also been suggested as the sixth ligand.^{1, 2}

Results and Discussion.—The proton NMR spectra at 220 Mc of ferricytochrome *c* and cyanoferricytochrome *c* are shown in Figure 2. Three parts of the spectra, which contain all the observed resonances, are reproduced with different vertical and horizontal scales. The spectrum of strongly overlapping resonances between 0 and -9 ppm comes from the bulk of the 650 protons of the polypeptide chain. The sharp lines between -4 and -6 ppm are the resonance of HDO and its side bands. The intensities of the resolved resonances observed in the regions 2 to 7 ppm and -10 to -35 ppm correspond to one to three protons. These resonances are shifted upfield or downfield by local magnetic fields arising both from aromatic ring currents^{4, 6} and from the unpaired electron of the heme iron.^{3, 7} The lines at -34.0, -31.4, and +23.2 ppm are the shifted resonances reported previously by Kowalsky.³

The resonances of amino acid residues located near the plane of an aromatic ring experience an upfield ring-current shift^{4, 6} which may be as large as 2 ppm for protons located within a few angstroms above or below the plane of a phenylalanine ring.⁹ Considerably larger shifts may result if the protons are located near several aromatic amino acid residues or near the plane of the heme group.⁸ Resonances of aliphatic amino acid residues can thus be shifted to positions several ppm upfield from DSS and may be well resolved at 220 Mc. Ring-cur-

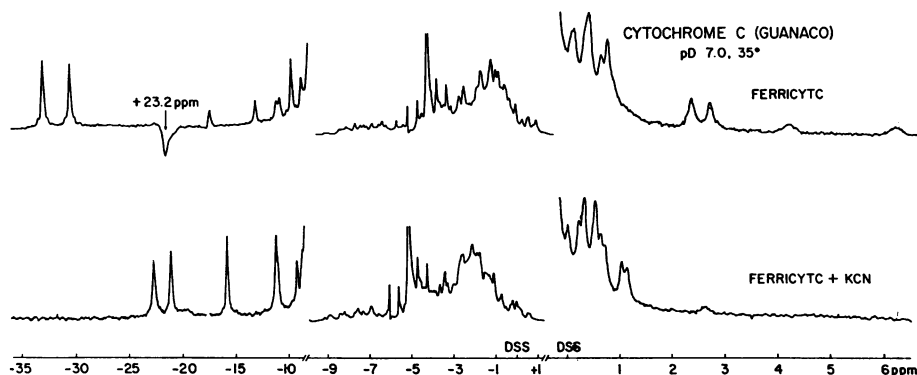


Fig. 2.—High-resolution proton NMR spectrum at 220 Mc of ferricytochrome *c* and cyanoferricytochrome *c*. No DSS was added to these samples. The sharp lines between -4 and -6 ppm correspond to the HDO resonance and its first and second spinning side bands. The vertical and horizontal scales are different for the three parts of the spectrum. The high-field line at $+23.2$ ppm is observed as an inversed resonance of the center band of the spectrum. (The HR-220 spectrometer operates with a 10 kc/sec field modulation. Usually one observes the first upfield side band. If large hyperfine shifts occur, parts of the center band and the different side bands of the spectrum overlap.)

rent shifts are very sensitive to the relative positions of the observed protons and the aromatic rings in the three-dimensional arrangement of the polypeptide chain and hence to conformational changes in the protein.^{4, 6} On the other hand, in the absence of conformational changes, ring-current shifts are independent of temperature.⁷

In the NMR spectra of paramagnetic heme proteins, one observes hyperfine shifts in addition to the ring-current shifted resonances.⁷ The unpaired electron of the iron in the low-spin ferric hemes (Fe^{3+} , $S = 1/2$) of ferricytochrome *c* and cyanoferricytochrome *c* is delocalized into the π -orbitals of the axial ligands and the porphyrin ring. Unpaired electron density is then transferred by spin polarization or hyperconjugation¹⁰ from the carbon or sulfur atoms to the protons attached directly, or in methyl and methylene groups (Fig. 1). The resulting contact shifts of the heme proton resonances are proportional to the spin densities on the nearest ring carbon atoms.¹⁰ It appears that for low-spin porphyrin iron (III) complexes contact shifts are large compared to pseudo-contact shifts.^{8, 11} Furthermore, because of the very short electronic relaxation times of low-spin ferric hemes,¹² the line widths of the proton resonances are essentially unaffected by electron-proton interactions. Hence, information about the unpaired electron distribution in the π -orbitals of the heme group can be obtained from NMR studies.⁷ For the following discussion it is of importance that hyperfine shifts are proportional to the reciprocal of temperature.

Ring-current shifts and hyperfine shifts can be distinguished from their temperature dependences.⁷ In the spectra of Figure 2, one finds that all the resonances in the regions -10 to -35 ppm and 2 to 7 ppm are shifted by hyperfine interactions.¹⁴ The temperature dependence of the ferricytochrome *c* spectrum between DSS and 3 ppm is shown in Figures 3 and 4. From Figure 4 it appears

most likely that the resonances of intensities three and six protons observed at +100 cps and +40 cps are shifted by ring-current fields. Further evidence for the presence of ring-current shifted lines between 0 and 3 ppm comes from the observation that the total intensity of the resonances outside 0 to -9.5 ppm (Fig. 2) corresponds to a larger number of protons than are on the ligands bound to the iron (Fig. 1). From their temperature dependences all the other reso-

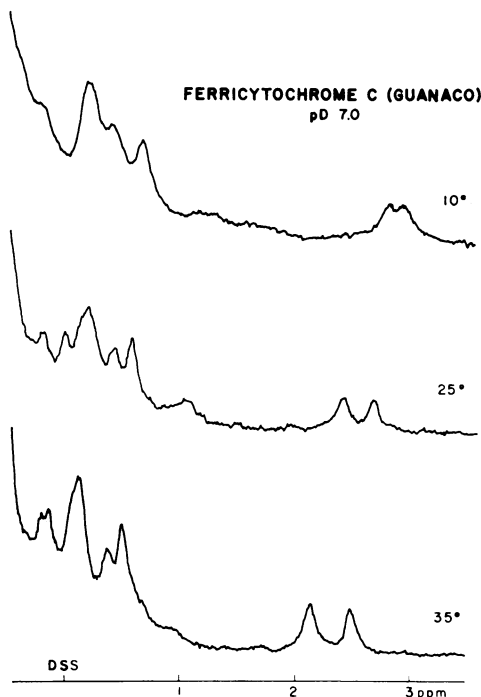


FIG. 3.—Dependence on temperature of the proton NMR spectrum at 220 Mc of ferricytochrome *c* between -1 and +3 ppm.

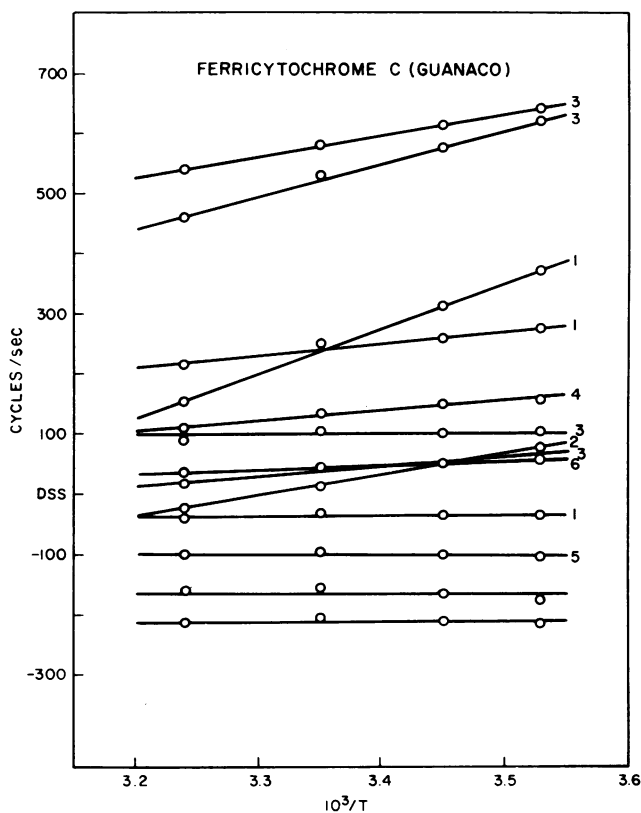
would expect to experience sizable contact shifts. An additional methylene resonance might be between 0 and -9 ppm.

At least five of the six methyl resonances between -34 and 2.5 ppm come from methyl groups of porphyrin *c* (Fig. 1).[‡] From the symmetry of the electronic wave functions of the heme group, and because no large negative spin densities would be expected on the carbon atoms of the porphyrin ring, it appears then extremely unlikely that any of the methyl or methylene resonances of the heme group could be shifted to +23 ppm. Furthermore no high-field resonances of intensity two or more protons have been observed above 5 ppm for any other low-spin ferric hemes¹¹ or heme proteins,^{7, 12, 15} including cyanoferricytochrome *c* (Fig. 2). This implies that the resonance at 3.2 ppm comes from one of the axial ligands. From previous work the sixth ligand is known to be a hemochrome-forming aliphatic amino acid residue.^{1, 2} Of these, only methionyl¹⁶ could con-

resonances upfield from DSS appear to be shifted by hyperfine interactions.

In the ferricytochrome *c* spectrum we then have the following hyperfine shifted resonances. At 35°C eight lines of intensity one proton are at -18.1, -13.8, -11.8, -11.5, 0.6, 1.0, 4.0, and 6.0 ppm (Figs. 2 and 3). Previous NMR investigations^{7, 11} showed that in low-spin ferric hemes and heme proteins the resonances of the methyl and methylene groups are not usually split into single proton resonances. Therefore the observed one-proton lines come most likely from the six single protons of porphyrin *c* and the 2,4-imidazole protons of the axial histidyl residue (Fig. 1). Six methyl resonances are at -34.0, -31.4, -10.3, 2.1, and 2.5 ppm (Fig. 2) and at 0.2 ppm (Fig. 4).[†] Resonances of two, four, and five protons at -0.1, 0.5, and 23.2 ppm (Figs. 2 and 4) would account in intensity for all but two of the remaining ligand protons in the structure of Figure 1, which one

FIG. 4.—Dependence on the reciprocal of temperature of the positions of the resonances of ferricytochrome *c* between -1 and $+3$ ppm. The number of protons corresponding to the intensities of the resonances is indicated on the right-hand side.



ceivably give rise to a contact shifted resonance of five protons, i.e., if the resonances of the methyl and the methylene groups next to the sulfur (Fig. 1) were accidentally degenerate. The shape of the resonance at 23.2 ppm does indeed indicate that it consists of at least two overlapping lines. Thus the NMR spectrum implies that the sixth ligand of the heme iron in ferricytochrome *c* is methionyl.

Analysis of the cyanoferricytochrome *c* spectrum¹⁴ indicates that the four ring methyls of the heme group (Fig. 1) are observed at -22.9 , -21.1 , -16.0 , and -11.4 ppm (Fig. 2). Most of the other hyperfine shifted lines are at high field from DSS, but no resonance of intensity more than one proton is above 2 ppm. As in ferricytochrome *c* it appears that there are three ring-current shifted methyl resonances between 0 and 1 ppm. The NMR spectrum between -10 and -35 ppm can be used for studies of the reaction of ferricytochrome *c* with cyanide ion. It was found that the cyanoferricytochrome *c* spectrum in Figure 2 corresponds to a 1:1 complex.¹⁴

In Figure 5 the high-field regions of the NMR spectra of ferricytochrome-*c*, cyanoferricytochrome *c*, and ferrocytochrome *c* are compared. The latter, which is diamagnetic (Fe^{2+} , $S = 0$), contains ring-current shifted resonances of intensity three protons at 3.3, 0.7, 0.6, and 0.6 ppm, a resonance of two protons at -0.1 ppm, and resonances of one proton at 3.7, 2.7, 1.9, 0.2, and -0.2 ppm

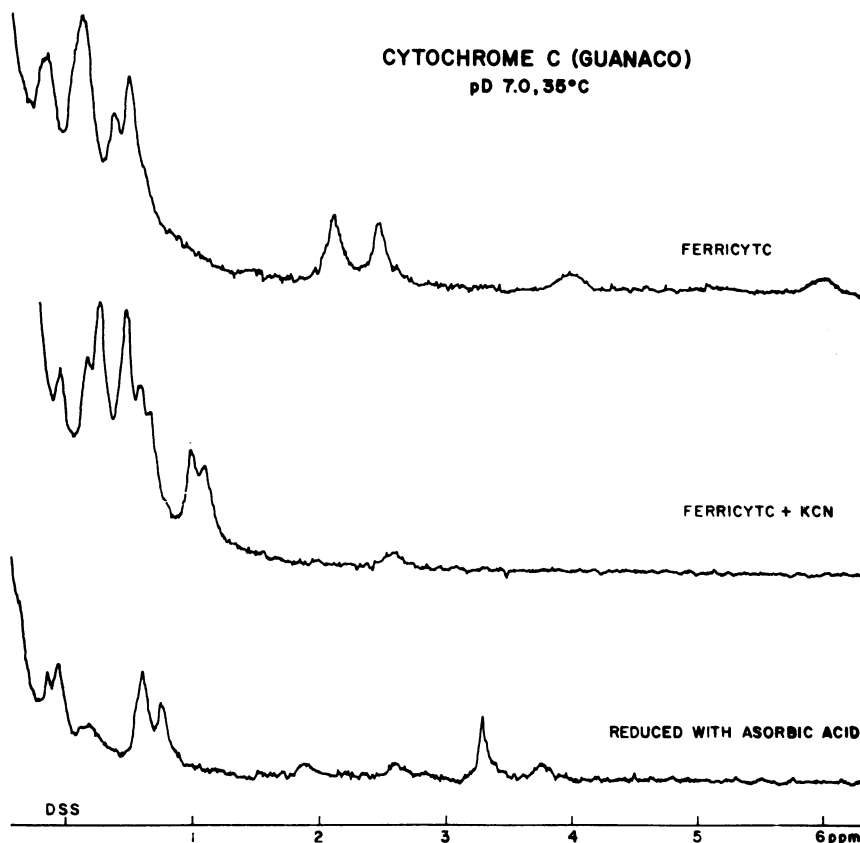
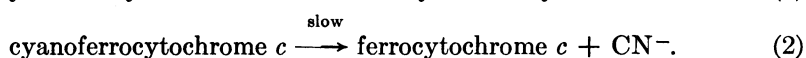
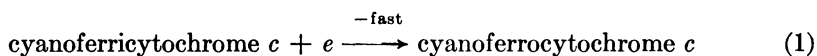


Fig. 5.—Proton NMR spectra between -1 and $+3$ ppm of ferricytochrome *c*, cyanoferricytochrome *c*, and ferrocytochrome *c*.

McDonald and Phillips⁵ suggested that the lines between 1.9 and 3.7 ppm come from the methyl group and three protons of the γ - and β -methylenes of an axial methionyl residue (Fig. 1) which would experience the strong ring-current field of the porphyrin ring. As discussed above, other explanations for the unusual positions of these resonances could be found. However, the following experiment implies that the assignment to the methionyl protons is correct.

A solution of cyanoferricytochrome *c* was reduced with dithionite. Figure 6 shows the resulting changes in the NMR spectrum. As judged from the disappearance of the hyperfine shifted resonances, the reduction was very fast at 9°C. On the other hand, the four resonances between 1.9 and 3.7 ppm of the ferrocytochrome *c* spectrum appeared very slowly. After 50 minutes, the reaction was not complete, as is seen from a comparison of the last two spectra of Figure 6. These observations agree with the following reaction mechanism proposed by George and Schejter¹⁷:



After the dissociation of the unstable cyanoferrocyanochrome *c* (2), the axial amino acid residue which was displaced by cyanide ion (Fig. 1) goes back into its place in the native protein. The data in Figure 6 show that the ferrocyanochrome *c* resonances between 1.9 and 3.7 ppm come from this axial ligand which then has to be methionyl, since this is the only hemochrome-forming amino acid residue^{1, 16} that contains a methyl group.

In addition to the identification of the sixth ligand, the NMR spectra yield data on the protein conformation and on the electronic structure of the heme group in ferri-cytochrome *c*. For example, a comparison of the spectra in Figure 5 shows that the ring-current shifted methyl resonances at 0.7, 0.6, and 0.6 ppm in ferrocyanochrome *c* are at different positions in both ferri-cytochrome *c* and cyanoferri-cytochrome *c*. Furthermore, it is seen from Figure 6 that two methyl resonances at 0.6 and 0.7 ppm are in identical positions in cyanoferrocyanochrome *c* and ferrocyanochrome *c*, whereas one methyl resonance moves from 0.6 to 0.7 ppm upon dissociation of the cyanide complex. This shows that at least minor conformational changes occur upon both inter-conversions between ferric and ferrous oxidation states and complex formation with cyanide ion. Extension of the analysis to the entire spectrum of the polypeptide chain will lead to a more detailed description of these conformational changes.

From Figure 2 the unpaired electron distribution in the heme group of ferri-cytochrome *c* differs greatly from that found in cyanoporphyrin iron (III) complexes.¹¹ Thus, as was found in other heme proteins,^{7, 15} the polypeptide-heme interactions have a strong influence on the electronic structure of the heme group. In particular, the comparison of ferri-cytochrome *c* and cyanoferri-cytochrome *c* implies that the coordination of the sixth ligand to the heme iron is an important factor. The proton resonances at -34.0 and -31.4 ppm† of ferri-cytochrome *c* correspond most likely to two ring methyls of the heme group.¹⁴ The two remaining ring methyl resonances are then much closer to -3.5 ppm, which is the resonance position of the ring methyls in diamagnetic porphyrins.⁸ This shows that there are large positive unpaired electron densities on ring carbon atoms of

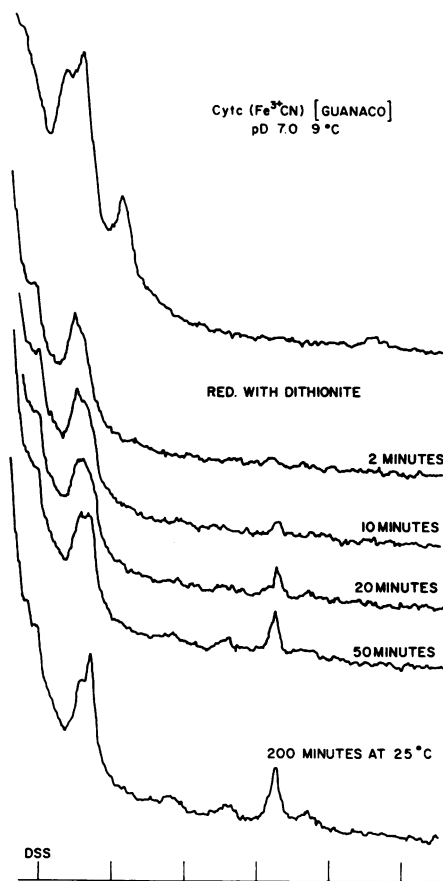


FIG. 6.—Proton NMR spectra between -1 and +3 ppm of cyanoferri-cytochrome *c* and of the reaction products observed at various times after reduction of cyanoferri-cytochrome *c* with dithionite.

two pyrrole rings of porphyrin *c*, and small positive or negative spin densities on the two other pyrroles. This is interesting because in its biological role ferricytochrome *c* takes up an electron. Since X-ray studies have shown that only one edge of porphyrin *c* is exposed to the solvent,² a possible path for the electron transfer would seem to be through this edge. Negative or small positive electron density at one of the exposed pyrrole rings might contribute toward a favorable free energy for the electron uptake.

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‡ In the methyl resonances at -34.0 and -31.4 ppm one observes a narrow and a broader component, which have approximately equal intensities. This arises most likely because the dipole-dipole coupling between the methyl protons is modulated by two different rotational motions—a very fast motion about the C—C bond from the porphyrin ring to the methyl group, and the slower rotation of the entire cytochrome *c* molecule. This effect was predicted from theoretical calculations by A. Redfield.¹³ To our knowledge it has not been observed previously in a protein NMR spectrum.

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